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EVALUATION OF A PURIFIED *SCHISTOSOMA*-SKIN TEST-ANTIGEN FOR THE DIAGNOSIS OF HUMAN BILHARZIA INFECTION

Report of Field Trials in Ethiopia, Rhodesia, Sierra Leone and Brasil

B. ENDERS, D. ECONOMY (1), K. D. HUNGERER and O. ZWISLER

SUMMARY

Using adult worms which were maintained for 24 hours in a sterile protein free medium in order to remove most of the host substances. *Schistosoma mansoni* antigens were isolated. These antigens were partially purified, standardized by chemical and immunological methods and preserved lyophilized in sterile and pyrogen-free form (*). To determine the specificity and sensitivity of this reagent field trials were carried out in selected areas of Ethiopia, Rhodesia and Brasil where Schistosomiasis mansoni shows high as well as low incidence, and in Sierra Leone where *S. haematobium* is prevalent. WHO Reference Skin Test Antigen served as a standard antigen. The results are compared with those from extensive stool examinations and were analysed by statistical methods. A total of 566 individuals (age over 10 years) invaded by *S. mansoni* has been skin tested and 94% gave a positive result according to the recommendation of the WHO. In 59 cases (age over 10 years) of *S. haematobium*-Bilharziasis the accuracy of the test was 95%.

(*) Schistosoma-Antigen for Bilharziosis Skin Test, Behring-Institute, Marburg/Lahn, West Germany.

INTRODUCTION

Schistosomiasis is one of the important human diseases which affects nearly 200 million people. This trematode infection represents a considerable clinical and public health problem in Asia, Africa and South and Middle America (West Indies). A world wide program for the fight against this parasitic disease is actively pursued through the encouragement and coordination of the World Health Organization. These activities include measures for control, epidemiologic and chemotherapeutic investigations and especially a program for standardization and evaluation of immunodiagnostic methods.

During the last years immunological tests were developed for individual diagnosis, epidemiological surveys and the evaluation of the efficacy of control. Based on a great number of publications (MAYER & PIFANO [13]; PELLEGRINO [15], and other Authors), KAGAN [5] mentioned that the intradermal test (IDT) for schistosomiasis has reached the stage of being an evaluated diagnostic technique.

Following the world wide program for standardization and evaluation of immunodiagnostic procedures in parasitic diseases it was our aim to develop a standardized schis-

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tosoma-skin test-antigen for the diagnosis of human schistosomiasis.

Patients with schistosomiasis show specific cutaneous sensitivity at an early stage of the disease. This skin sensitivity may result from the formation of circulating antibodies (TALIAFERRO & TALIAFERRO [20]; GUERRA et al. [4]; PELLEGRINO et al. [17]; KAGAN & PELLEGRINO [7]). When sensitized individuals receive an intradermal injection of an extract from adult schistosoma worms or cercaria, the skin reactions produced 15 minutes after injection are usually of the histaminic or immediate type. This skin test has distinct advantages since it is relatively inexpressive, permits screening of large population groups in a relatively short time, provides immediate results and thus is a valuable aid in epidemiologic investigations and public health practices, (SADUN [19]).

In spite of the relatively wide use, the intradermal test for schistosomiasis has some disadvantages and limitations which must be taken into consideration to interprete the results. The skin reactivity becomes evident one to two months after infection and persists for the duration of the disease and even for some years after effective treatment. Therefore IDT is not suitable for the supervision of chemotherapeutic cure and treatment should not be based on a positive skin test only, unless parasitologic and other serologic studies verify active schistosomiasis. On the other hand, it is known that exposure of individuals to cercariae of non human schistosomes e.g. *Trichobilharzia* spp., *Ocellata* spp. etc. (MOORE et al. [14]; HOHORST et al.[3]), will induce reactivity in the intradermal test when antigens from human schistosomes are injected.

Even this test is a measure of recent and past experience with schistosoma cercariae, but it remains anyhow a useful tool for epidemiological studies provided the concomitant presence of non human schistosomes can be excluded (FIFE [2]).

MATERIAL AND METHODS

The antigen used up to now prepared from adult schistosomes is a complex mixture of various nitrogen-containing components, those as e.g. antigenic components of the parasite or host substances which are incorporated in the tegument (mimicry and self-defense) or which are located in the intestinum of the worm. For a precise standardization of the skin test-antigen on a chemical basis it is necessary to remove the host substances as far as possible. The living worms obtained by liver perfusion of infected mice, were cultivated under sterile conditions for 24 hours in a protein-free medium. After this first step of purification and the subsequent isolation of the antigenic components the antigen can be adjusted to contain a defined amount of nitrogen. This antigen is standardized with regard to its nitrogen content (0.03 mg N/ml) because PELLEGRINO and coworkers [17] found a linear relationship between the logarithm of the antigen nitrogen concentration and the average area of the skin test induration.

In addition to this chemical standardization method we developed a satisfactory immunological method based on the positive cutaneous anaphylaxis (PCA) in guinea-pigs. 1 ml of a pool of sera derived from clinically proven bilharzia cases or a standard schistosoma hyperimmune serum from rabbits is injected intravenously. After 48 hours several concentrations of antigen (0.1 ml) are injected intradermally in the appropriate places of the shaved test animals. 20 minutes after intradermal antigen injection Evans blue is given intravenously. The antigen will react with the anaphylactic antibodies fixed onto the tissues. A blue spot indicates a positive reaction.

The serologic activity of the schistosoma-skin test-antigen is also examined by the indirect hemagglutination test. Both immunological tests for standardization are performed by using a pool of sera derived from clinically proven bilharzia cases and bilharzia specific hyperimmune sera from rabbits.

With the development of this modified method for the isolation and purification and the introduction of improved immunologic techniques besides the chemical analysis for standardization of a specific schistosoma-skin test-antigen we hope that this antigen will be

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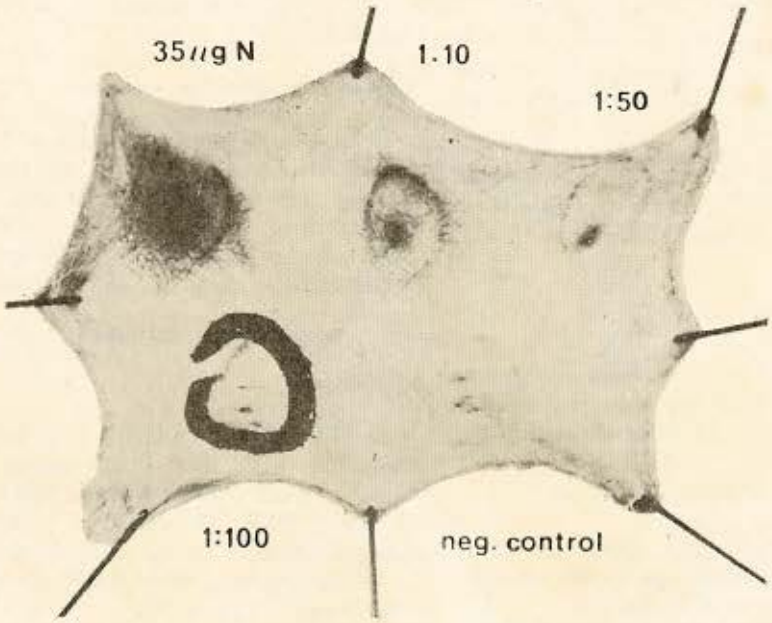

PCA—reaction for biological standardization of BILHARZIA INTRADERMAL TEST ANTIGEN

a valuable tool for the diagnosis of schistosomiasis.

a) *Schistosoma* antigen for bilharziosis-skin test prepared as follows: Adult *S. mansoni* obtained from experimentally infected mice are incubated for 24 hours in a sterile protein-free medium to remove most of the host substance from the intestinal tract of the parasites. The washed worms are lyophilized, delipidisized (–70°C, ether) and ultrasonically homogenized in BBS (isotonic borate buffered saline, pH 7.4, 1:10 000 Merthiolate). After over-night incubation (4°C, shaking) the homogenate is centrifuged (4°C, 20 000 x g, 1 hour), the supernatant is dialysed for 24 hours against BBS, adjusted to 0.03 mg N/ml, and the immunological reactivity of the antigen is checked by in vitro and in vivo methods (IHA: indirect hemagglutination test and PCA: passive cutaneous anaphylaxis).

After filtration and lyophilization the antigen is sterile and free of pyrogens. Pyrogene test is carried out in rabbits. Prior to use the antigen has to be reconstituted to give an isotonic solution. The solvent BBS serves as control antigen.

b) "WHO Reference Skin Test Antigen": Melcher's sterile, acid soluble protein fraction of adult worms *S. mansoni* adjusted so as to contain 0.028 mg N/ml, 1:5 000 Merthiolate).

c) Melcher's schistosoma-skin test-antigen: prepared of adult *S. mansoni*, adjusted to 0.012 mg N/ml, liquid. Ministry of Health, Rhodesia.

Clinical material

In the first study we present the results of the comparison of two skin test-antigens (schistosoma antigen for bilharziosis skin test (Behringwerke AG) and WHO reference antigen) in an extensive survey in

a) **Ethiopia:** in two areas which for many years have been known as to be endemic (LEMMA [12]; BUCK et al.[1]; LEMMA [11]). The study was conducted in the Wonji Sugar Estate on the Awash river and in Adwa, a highly endemic area in the northern part of the country. The infective agent in both areas is *Schistosoma mansoni* exclusively.

b) **Brasil:** in Catinga do Moura, a *Schistosoma mansoni* endemic area near Bahia.

c) **Rhodesia:** the comparison of the antigen produced by our laboratory and the reference antigen of the Ministry of Health, Rhodesia, was performed in an area of Rhodesia where *Schistosoma mansoni* causes schistosomiasis.

d) **Sierra Leone:** the sensibility of the schistosoma antigen for bilharziosis skin test (Behringwerke AG) in patients suffering by *Schistosoma haematobium* bilharziosis was carried out in the area of Mobei with high incidence of this parasite.

Performance and interpretation of the test

The test was performed following the recommendations of WHO: Exactly 0.05 ml each of the antigen and antigen control were injected strictly intradermally in the flexor surface of the right and left forearms. Results were read after 15 minutes. The wheals were recorded and their areas measured by the WHO Standard Stencil recommended for this test.

Methods of parasitological examination

Stool specimens were collected and examined for ova by direct smear. In the case of positive skin test reactions but negative stool examinations two additional concentration methods were performed: the Teleman technique and the miracidia hatching test.

RESULTS AND STATISTICAL ASSAY

a) Ethiopia

In Wonji 303 persons of both sexes were examined. The most highly exposed group were the field workers with an infection rate of about 20% (LEMMA [11]). The second group consisted of school children aged 10-15. In this latter group about 3-5% positive cases were found (LEMMA [11]).

In Adwa where the infection rate is estimated to be about 80-90% (BUCK et al.[1]; LEMMA [12]), 266 children and adults ranging in age from 5-50 years were tested.

In both areas the parasitological examinations of all groups, independent of age, produced a total of 239 positive *S. mansoni* cases (Wonji 106, Adwa 133). Those persons who were thought not to be frequently exposed to infective agents and who were under medical care, i.e. laboratory personnel and school children, were selected to act as a group of negative cases and served as controls. Those individuals whose stools were free of ova at several examinations and who subsequently had no skin reaction with both antigens served as negative standard for the statistical analysis.

A total of 434 male and 135 female test persons, all more than 5 years old, were examined. The percentage distribution of all skin test indurations of the whole material investigated, divided according to sex and antigen applied, is given in the upper parts of Figs. 1 and 2. These graphs show that in respect to the response to the test antigen the reactions can be divided into two clearly distinguishable groups, whereas no remarkable difference between the BW-test antigen and the reference antigen was observed.

The curves below in Fig. 1 and 2 represent the frequency of skin test indurations in cases of active schistosomiasis (diagnosis based on identification of ova in stool specimen). Hence, it follows that peak 1 between 0.1 cm^2 and 1 cm^2 in Figs. 1 and 2 indicates the skin reaction of non-infected persons since this peak is absent in the graphs of infected persons who were recognized only by positive stool tests. The irregularity of the curves in the groups with active schistosomiasis is not significant since the statistical assay described below results in a normal distribution of the skin test indurations in the individual groups.

As expected, the cumulative frequency of the skin test wheals in all test persons does not show a normal distribution. The bimodality of the curves is explained by the inclusion of both, bilharziosis positive and negative test persons, in the material investigated. The cumulative frequency curve of the persons whose stool tests were positive as well as the results from the probit analysis are given in Fig. 3.

With the exception of a somewhat irregular frequency curve of female patients with positive stools, tested with the reference antigen, the cumulative frequency was homogenous. The probit analysis indicates that the large-sized indurations do not fall within the normal distribution. Such a hypersensitivity, however, was observed in a few cases with positive stool only and does not influence the positive results of this test.

The analogous mathematical evaluation of the findings in the group considered by us as bilharziosis negative shows also normal distribution of the wheal sizes. These results indicate that the size of the indurations depends exclusively on the infection or non-infection with bilharziosis which signifies the high specificity of the antigen used. Previous work has demonstrated that a wheal area of 1.0 cm^2 which is caused by the reference antigen must be regarded as the lower limit for a positive interpretation of the skin test (WHO recommendation). In accordance with the above discriminating limit the indurations measured by us and given in Table I can be divided into three groups. It can be concluded that the use of

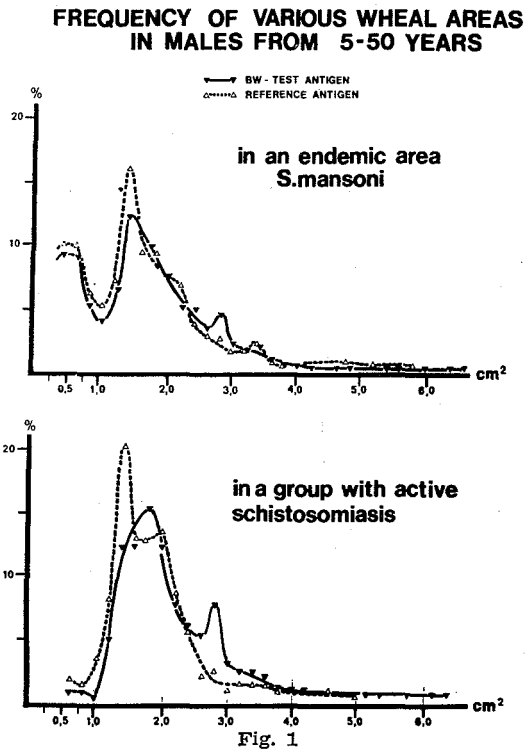

Fig. 1

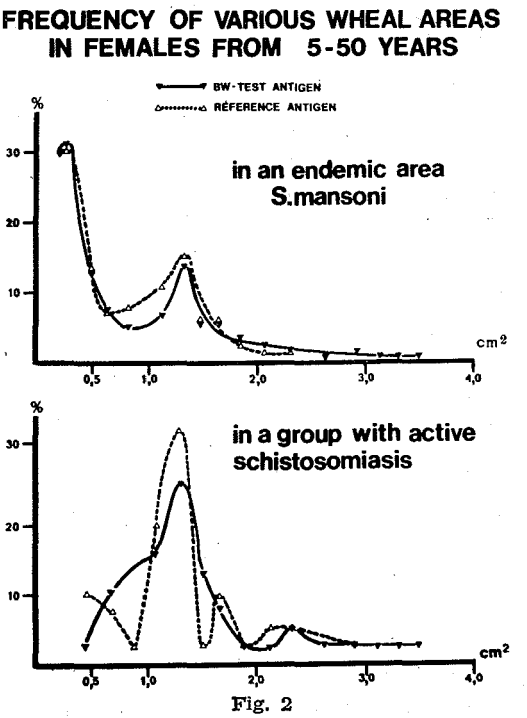

Fig. 2

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COMPARATIVE STATISTICAL ANALYSIS OF TWO SKINTEST ANTIGENS IN PROVEN CASES OF S.MANSONI INFECTION

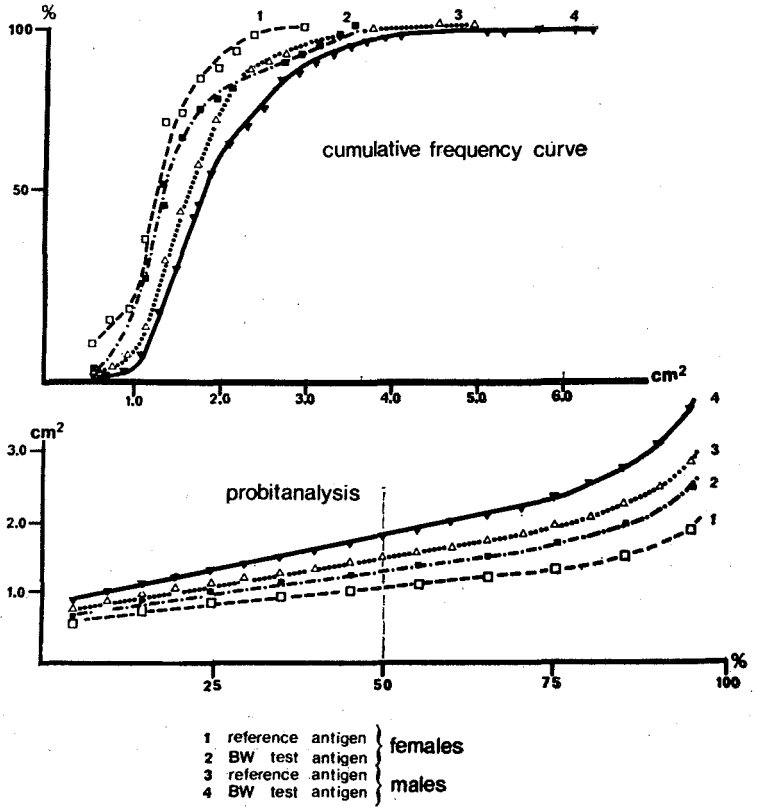

Fig. 3

TABLE I

Reactivity of two skintest antigens

S. MANSONI

PATIENTS	SKIN-REACTION cm²	REACTIVITY %			
		BW-test-antigen		reference-antigen	
		male	female	male	female
active Schistosomiasis stool positive 10–50 years	0,1 – 0,4	0,0	0,0	0,0	7,7
	0,5 – 0,9	1,9	11,5	7,0	11,5
	>1,0	98,1	88,5	93,0	80,8
negative controls >5 years	0,1 – 0,4	66,7	86,5	60,7	88,5
	0,5 – 0,9	33,3	13,5	39,3	11,5
	>1,0	0,0	0,0	0,0	0,0

these limits does not result in false positive interpretations in the case of negative control persons tested with both, the BW-test antigen and the reference antigen.

On the other hand, in cases of definite bilharziosis (positive stool test) in persons above the age of 10 the WHO discriminating limit recognized as non-infected 7% of the male and 19.2% of the female cases tested with the reference antigen, and 1.9% of the male and 11.5% of the female cases tested with BW-test antigen. These findings call for a tentative new definition of the discrimination limits on the basis of a statistical assay (Table II).

TABLE II

Comparative statistical analysis of two skintest antigens

PATIENTS	sex	number	SKINREACTION cm²					
			mean ± s_m range 99%		mean probitanalysis		normal distribution probability 95%	
			BW-TEST ANTIGEN	REFERENCE ANTIGEN	BW-TEST ANTIGEN	REFERENCE ANTIGEN	BW-TEST ANTIGEN	REFERENCE ANTIGEN
active Schistosomiasis	M	155	2.0±0.13	1.6±0.10	1.76	1.46	0.7-3.3	0.6-2.7
stool positive 10-50 years	F	26	1.4±0.20	1.2±0.20	1.29	1.06	0.5-2.2	0.4-1.9
negative controls	M	84	0.4±0.05	0.4±0.05	0.33	0.34	0.08-0.7	0.07-0.8
>5 years	F	52	0.3±0.04	0.3±0.05	0.27	0.28	0.1-0.6	0.05-0.6

This attempt revealed that the reference antigen does not provide an unequivocal limit since in a normal distribution with a probability of 95%, the lower limit covering the positive male cases is 0.6 cm² and the upper limit covering the negative male cases is 0.8 cm², the corresponding limits for female patients being 0.4 cm² and 0.6 cm² respectively.

The corresponding values for our test antigen in male test persons show a clear limiting value of 0.7 cm² between the positive and negative groups. The limits of the female groups overlap by 0.1 cm² only (0.1-0.6 cm² and 0.5-2.2 cm²). This comparative mathematical evaluation of the induration sizes exhibits a higher specificity and sensitivity of the antigen produced in our laboratories by the new method.

Furthermore, the records were examined to find which group of test persons falls within the range of 0.5-0.9 cm²: no adult with proved schistosomiasis had such a small induration, and only children of both sexes with proven bilharziosis (positive stool test) developed indurations in this range. This applies to 11% of all boys aged 5-11 and to 15% of all girls aged 5-12.

Similar findings were worked out by PELLEGRINO et al.[16]. The ID-test did not give favourable results in children when the test was performed on the forearm. By a comparative application of the skin test-antigen in the arm and the back a higher sensitivity was found in the back. We were able to verify these findings in a few cases of stool positive boys and girls who showed a negative (< 1.0) skin reaction in the forearm and

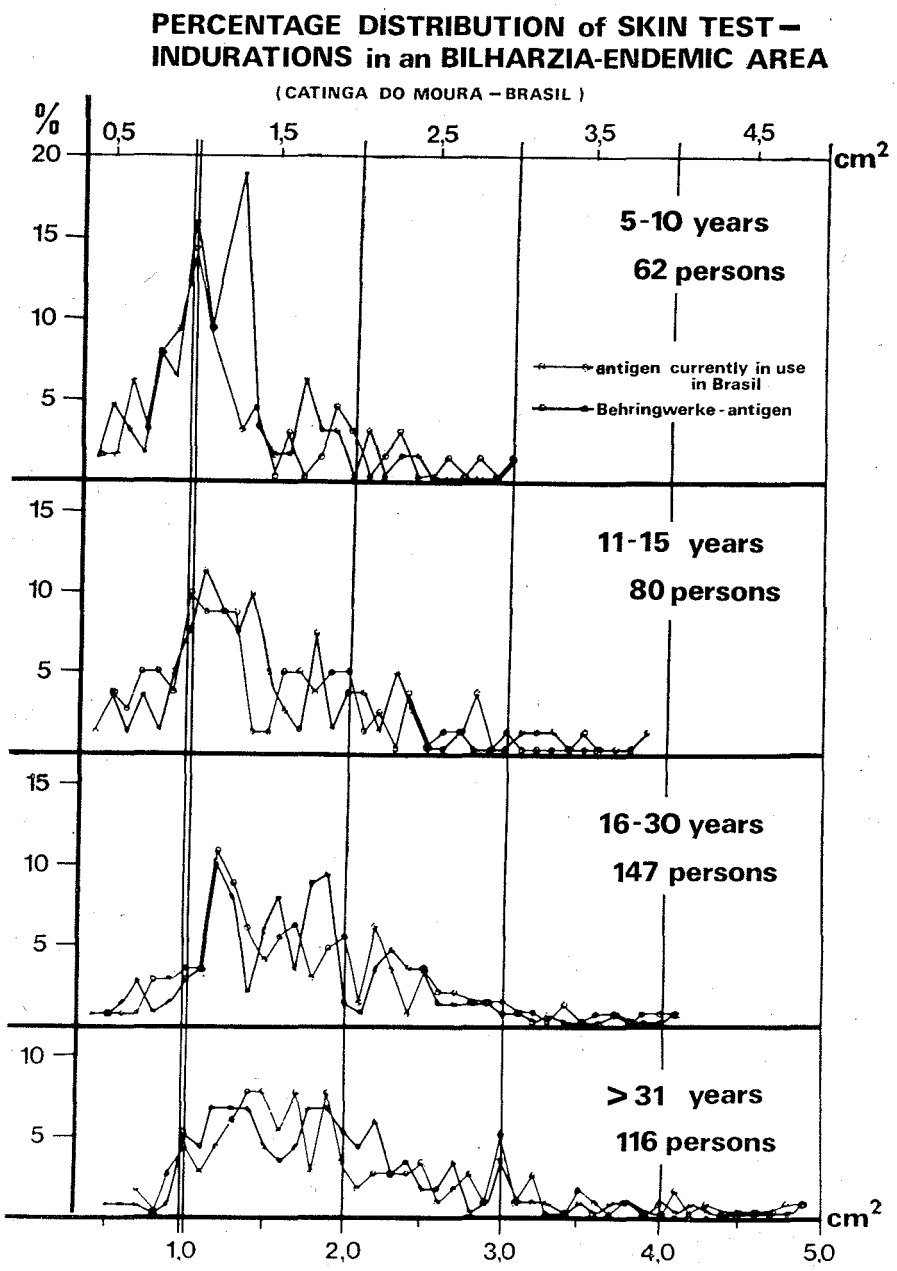
PERCENTAGE DISTRIBUTION of SKIN TEST – INDURATIONS in an BILHARZIA-ENDEMIC AREA
(CATINGA DO MOURA – BRASIL)
%
0,5
1,5
2,5
3,5
4,5
cm²
5-10 years
62 persons
antigen currently in use in Brasil
Behringwerke-antigen
11-15 years
80 persons
16-30 years
147 persons
> 31 years
116 persons
1,0
2,0
3,0
4,0
5,0

Fig. 4

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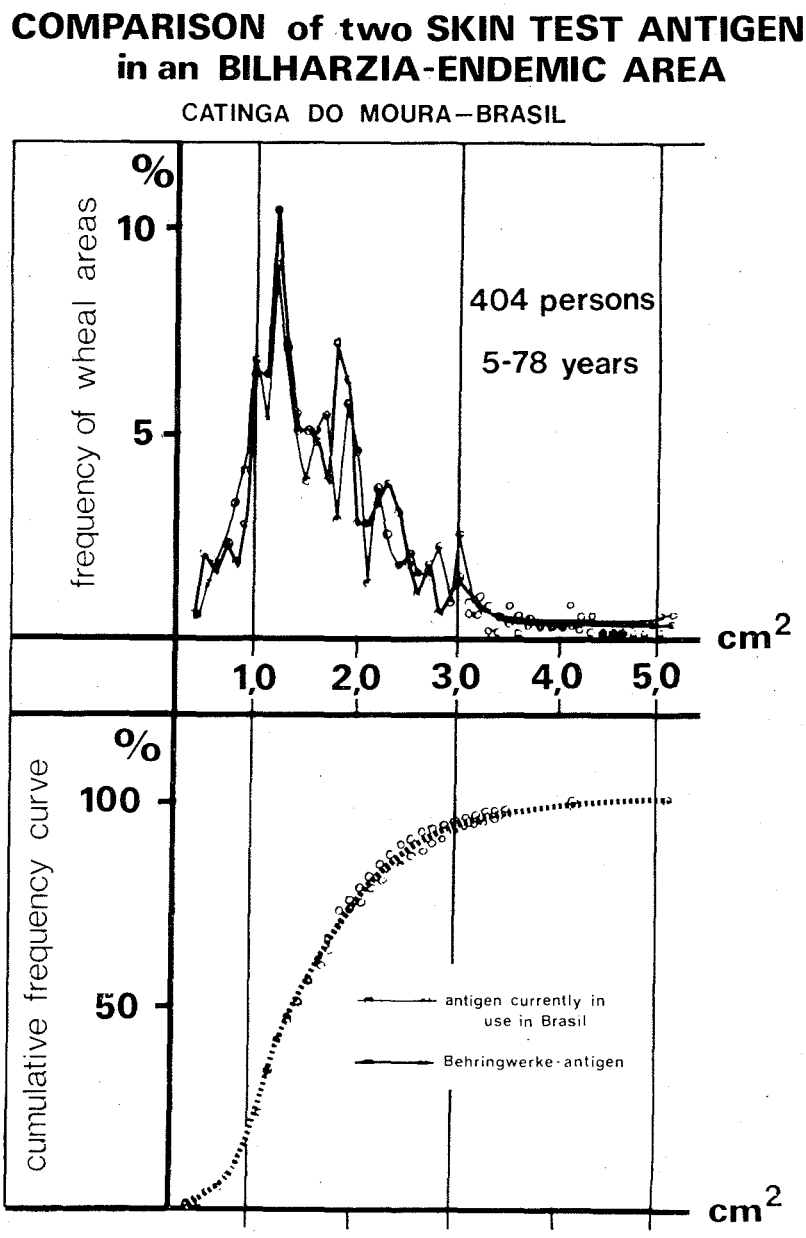

Fig. 5

ENDERS, B.; ECONOMY, D.; HUNGERER, K. D. & ZWISLER, O. — Evaluation of a purified *Schistosoma*-skin test-antigen for the diagnosis of human bilharzia infection. Report of field trials in Ethiopia, Rhodesia, Sierra Leone and Brasil. *Rev. Inst. Med. trop. São Paulo* **16**:305-316, 1974.

Frequency of various skin test indurations in patients with active schistosomiasis (Rhodesia)

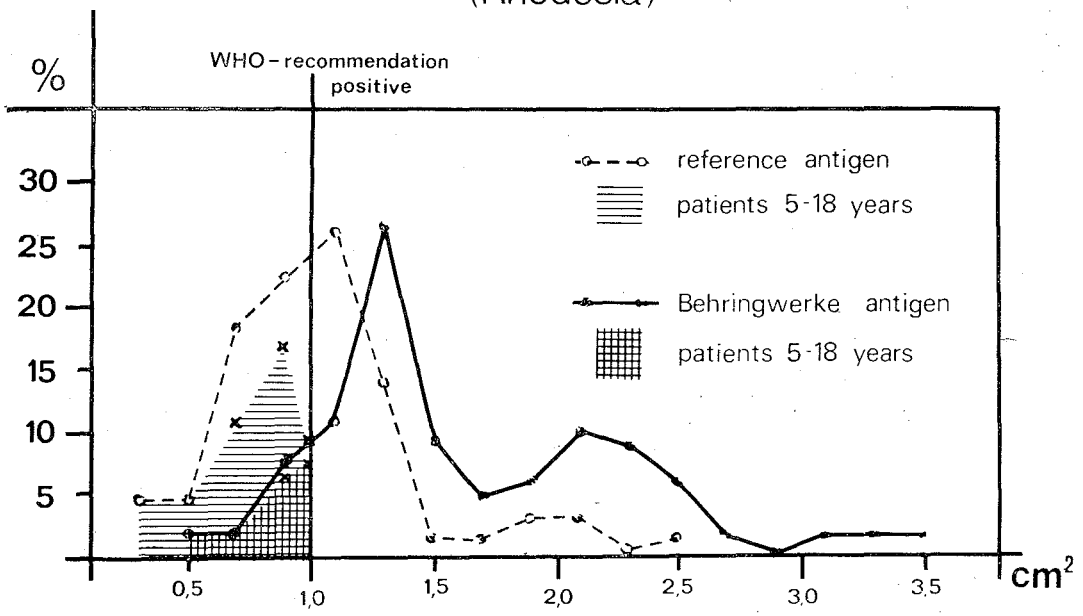

Fig. 6

Frequency of various skin test indurations in patients with active Schistosoma haematobium infection (Sierra Leone)

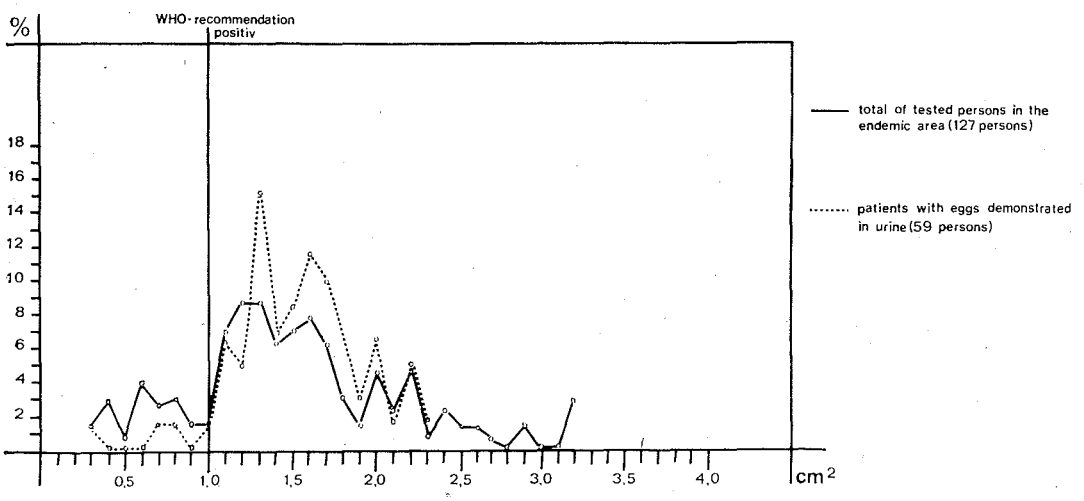

Fig. 7

more than 1.0 cm² in the back. Therefore, we propose the back as the best place for performing the skin test in children up to the age of 12.

Finally, it becomes evident that for future applications of the BW-test antigen the discrimination limits should be defined as follows:

0.1 — 0.4 cm²	=	negative
0.5 — 0.9 cm²	=	uncertain (in the case of children up to the age of 12, otherwise negative)
1.0 cm² and above	=	positive

b) Brasil

It has been recognized that the intensity of the reactions is greater in adults than in children (MAYER & PIFANO [13]) and is more pronounced in males than in females. KLOETZEL & R. DA SILVA [10] postulated in their study on the development of skin reactivity that the bilharzia specific skin sensitivity develops slowly in individuals living in endemic areas. The lower skin reactivity of children apparently is related to the length of time spent in an endemic area rather than to immunological incompetency.

The study carried out by Dr. N. KENT (WHO, Geneva) in Catinga do Moura using skin test antigen prepared in our laboratory and the antigen currently in use in Brasil verifies these results.

The comparison of the percentage distribution of the skin test indurations according to age shows exactly that children before puberty have a lower percentage of positive reactions compared with adults. These data are in accordance with those given by WHO.

The analogous mathematical evaluation of the findings in Brasil also shows a normal distribution of the wheal sizes and no significant difference between the antigens tested.

c) Rhodesia

The small survey carried out in an area in Rhodesia where *S. mansoni* is endemic, also demonstrates that patients who show negative skin tests but are suffering from active schistosomiasis are primarily young people.

d) Sierra Leone

PI-SUNYER and coworkers [18], KHALIL [9] and other Authors reported that the IDT was highly sensitive (93-96%) in patients passing eggs of *S. haematobium.*

The findings of a small skin test study in Sierra Leone with *S. mansoni* antigen prepared in our laboratories confirm that in areas with only *S. haematobium* an intradermal survey also may be of use.

In a group of 59 infected persons the skin test of 95% was positive.

RESUMO

Avaliação de antígeno purificado para o diagnóstico da esquistossomose humana através de prova intradérmica

Antígenos de *Schistosoma mansoni* foram produzidos a partir de vermes adultos mantidos por 24 horas em meio estéril isento de proteínas a fim de remover o máximo de substâncias do hospedeiro. Esses antígenos foram purificados parcialmente, padronizados através de métodos químico-imunológicos e conservados liofilizados, sob forma estéril e isenta de resíduos pirogênicos (*). Com o objetivo de determinar a especificidade e sensibilidade do reagente, realizaram-se pesquisas de campo em áreas selecionadas da Etiópia, Rhodésia e Brasil, países em que há incidência, tanto elevada como reduzida, de esquistossomose mansônica, e em Sierra Leone onde há prevalência do *S. haematobium.* Como padrão, empregou-se o antígeno de referência da O.M.S. para provas intradérmicas. Os resultados foram comparados aos de exames de fezes realizados em larga escala, e analisados por métodos estatísticos. Submeteram-se à prova intradérmica 566 indivíduos (de grupos etários superiores a 10

(*) Antígeno esquistossômico para prova intradérmica de bilharziose.

anos) infestados pelo *S. mansoni* e 94% apresentaram resultado positivo, segundo os critérios da O.M.S. Em 59 casos (grupos etários a superiores a 10 anos) de bilharzíase pelo *S. haematobium* a precisão da prova atingiu 95%.

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